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Review Letter

The measurement of free radical reactions in humans

Some thoughts for future experimentation

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The question as to whether free radical reactions are a major *cause* of tissue injury in human disease, or merely an accompaniment to such injury, is very difficult to answer because of lack of adequate experimental techniques. New techniques that are becoming available are discussed, with specific reference to their use in humans.

Free radical; Hydroxyl radical; Iron; Lipid peroxidation; Oxidative stress; Fenton reaction

1. INTRODUCTION

There is a massive literature concerning the role of free radical reactions in human disease; table 1 lists some of the conditions in which free radical involvement has been proposed. A question arises as to whether radical production in many diseases is a major *cause* of tissue injury or merely a *consequence* of it [1,2,64,65]. Attempts to answer this question are currently hampered by lack of adequate methodology, although the reports of Ames et al. [31,32] that bacterial cells respond rapidly to oxidative stress by synthesis of specific 'alarmone' molecules may be of great importance in the future, provided that such molecules are also made in animal cells.

A common current approach is to try to measure end-products of lipid peroxidation in human body fluids or tissue extracts. Unfortunately, the commonly used 'TBA test' is unspecific and can give misleading results [3,12] and measurement of UV-absorbing 'diene conjugates' may in fact be measuring something in human fluids that does not arise by lipid peroxidation [4]. Corongiu et al. [5] have introduced an interesting modification of the diene conjugate method, but its applicability to human body fluids is presently unclear [4]. Analysis of polyunsaturated fatty acid changes in membrane lipids often gives interesting results, but many diseases and toxins alter the activity of fatty acid synthetase/desaturase enzymes, changing the rate of membrane lipid turnover. Determination of specific peroxidation end-products, arising from such cytotoxic aldehydes as 4-hydroxynonenal, may prove in future to be an excellent assay method [7,8], as may enzymic determination of lipid peroxides [9]. Hydrocarbon gas exhalation [10,60] is a useful assay for animals, but very difficult to apply to humans except under carefully controlled laboratory conditions, and so not suitable for routine studies. Light emission shows considerable promise as an assay of lipid peroxidation, and perhaps other free radical reactions, in cells and perfused organs [11], but again might prove difficult to apply to humans, although the pace of advance in fibre-optic technology is so great that one cannot rule it out.

One problem in relying on measurements of lipid peroxidation as an index of free radical reac-

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Table 1

Some clinical conditions in which the involvement of oxygen radicals has been suggested

Inflammatory-immune injury
Glomerulonephritis (idiopathic, membranous)
Vasculitis (hepatitis B virus, drugs)
Autoimmune diseases
Rheumatoid arthritis

Ischemia – reflow states
Stroke/myocardial infarction
Organ transplantation

Drug- and toxin-induced reactions

Iron overload
Idiopathic haemochromatosis
Bantu tribe iron pot beer drinkers
Thalassaemia and other chronic anaemias treated with multiple blood transfusions
Nutritional deficiencies (Kwashiorkor)

Alcoholism

Radiation injury

Ageing
Disorders of 'premature ageing'

Red blood cells
Phenylhydrazine
Primaquine, related drugs
Lead poisoning
Protoporphyrin photo-oxidation
Malaria
Sickle cell anaemia
Favism
Fanconi's anaemia

Lung
Cigarette-smoke effect
Emphysema
Hyperoxia
Bronchopulmonary dysplasia
Oxidant pollutants (O_3)
ARDS (some forms)
Mineral dust pneumoconiosis
Bleomycin toxicity
SO_2 toxicity

Heart and cardiovascular system
Alcohol cardiomyopathy
Keshan disease (selenium deficiency)
Atherosclerosis
Adriamycin cardiotoxicity

Kidney
Autoimmune nephrotic syndromes
Aminoglycoside nephrotoxicity
Heavy metal nephrotoxicity

Gastrointestinal tract
Endotoxin liver injury
Halogenated hydrocarbon liver injury (e.g. bromobenzene, CCl_4, halothane)
Diabetogenic action of alloxan
FFA-induced pancreatitis
NSAID-induced G + I tract lesions
Oral iron poisoning

Brain
Hyperbaric oxygen
Vitamin E deficiency
Neurotoxins
Parkinson's disease
Hypertensive cerebrovascular injury
Neuronal ceroid lipofuscinoses
Allergic encephalomyelitis and other demyelinating diseases
Aluminium overload
Potentiation of traumatic injury

Eye
Cataractogenesis
Ocular haemorrhage
Degenerative retinal damage
Retinopathy of prematurity
Photic retinopathy

Skin
Solar radiation
Thermal injury
Porphyria
Hypericin, other photosensitizers
Contact dermatitis

tions is that, in most (not all [22]) cases of 'oxidative stress', lipid peroxidation is probably *not* the major mechanism by which increased generation of oxygen-derived species causes primary cellular injury. For example, the toxicity of paraquat to lung involves O_2^- and H_2O_2, but the tissue damage may be mediated by depletion of nicotinamide nucleotides and/or inhibition of lipid synthesis rather than by lipid peroxidation ([13] loc cit.) Similarly, lipid peroxidation occurs at a late stage in paraquat-treated plants; the primary mechanism of damage seems to be direct inactivation of Calvin cycle enzymes by increased H_2O_2 formation [14]. Inhibition of lipid peroxidation by antioxidants does not abolish the toxicity of MPP^+ [15], mercury [16] or thiol-modifying reagents [17] to hepatocytes, nor can the toxicity of H_2O_2 [29], paracetamol [55], diquat [19], hyperoxia [19] or, possibly [61], bromobenzene [18] to these cells be attributed to lipid peroxidation. Excessive GSH depletion [19,29] and/or DNA damage [20], perhaps leading to lethal depletions of nicotinamide nucleotides as poly(ADP-ribose) synthetase is activated [21,30], and/or to impairments in the regulation of intracellular Ca^{2+} concentration, may be the primary toxic mechanisms.

Poisonous end-products of lipid peroxidation, such as unsaturated aldehydes, may be of some importance in spreading injury away from the cells first damaged [22,56,59], but measuring such end-products may be looking at something that is remote from what triggered the injury initially. Hence it can be argued that studying lipid peroxidation in whole subjects will not lead to a resolution of the 'cause or consequence' question posed at the beginning of this article, since lipid peroxidation is usually a late event. In any case, the methods most frequently used currently are probably inadequate (see above) to track accurately the rate of peroxidation in the whole body.

2. WHAT CAUSES CELLULAR DAMAGE?

The cellular injury produced by hyperoxia and redox-cycling drugs involves increased generation of superoxide radical (O_2^-) and H_2O_2 ([28] loc cit.). Both of these have some direct damaging effects [28] and H_2O_2 can deplete GSH as it is metabolized by glutathione peroxidase, but we believe that a key mechanism of toxicity is the metal-ion-dependent formation of highly reactive hydroxyl radical, $^{\cdot}OH$, or a species of similar reactivity [2,23]. In the absence of precise chemical information as to what such an alternative species might be, we shall continue to refer to $^{\cdot}OH$, although the argument would not be altered if "$^{\cdot}OH$' turned out to be a different reactive radical. Hence the location of metal-ion complexes capable of causing $^{\cdot}OH$ radical formation will influence the type of damage that is done by oxidative stress [23,24]; DNA seems often to have such metal complexes attached to it in vivo [20,63] and so oxidative stress may lead to DNA damage and consequent lethal nicotinamide nucleotide depletion (see above). Thus chelating agents, such as desferrioxamine [25], that are capable of preventing metal-dependent $^{\cdot}OH$ radical generation can minimize damage by O_2^- and H_2O_2, both in cellular systems (e.g. [19,20,37]) and in whole animals (reviewed in [23,24]). It must be recognised that chelating agents such as desferrioxamine may have multiple mechanisms of action [23–26] and often cannot get to the correct sites [20]. Desferrioxamine itself is unlikely to be of use for human therapy [27] and more promising agents exist [58], but the significant protective effects of desferrioxamine observed in whole animal models of human disease are *consistent* with our view [2,23–25] that metal-dependent $^{\cdot}OH$ formation is an important cytotoxic mechanism and does contribute significantly to tissue damage in several human diseases. Superoxide dismutase and H_2O_2-removing systems might also be of therapeutic importance, as may inhibitors of radical-generating enzymes (reviewed in [57,58]).

3. CAN HYDROXYL RADICAL BE MEASURED IN VIVO?

3.1. *Principles*

Hydroxyl radical is so highly reactive that measuring its formation in vivo will be correspondingly difficult. Its formation cannot be inferred from protective effects of "$^{\cdot}OH$ scavengers' (such as dimethyl sulphoxide, thiourea or dimethylthiourea) in animal models of human disease, since these molecules react with many other species. For example, thiourea combines with H_2O_2 and scavenges hypochlorous acid produced by the myeloperoxidase system [33]. At

present, only mannitol and formate remain as specific scavengers of ·OH, but formate is metabolised rapidly in vivo to CO_2 by formate dehydrogenases and the peroxidatic action of catalase, whereas mannitol does not enter cells easily. Several in vitro assays for ·OH, such as dimethyl sulphoxide oxidation [35,36], ethylene formation [34,36] or spin-trapping [38–40] do not seem applicable for use to detect ·OH in humans, although they may be of value for isolated cells and, perhaps, for perfused organs, and spin-traps can detect carbon-centred radicals in whole animals ([62] loc cit.).

3.2. *DNA damage*

If DNA is an important cellular target for attack by ·OH or related radical species, can the products of attack be measured? This approach was pioneered by Ames et al. [41], who measured thymine and thymidine glycol, as products of oxidative DNA damage, in human urine. The assay method in [41] is very tedious however, although measurement of hydroxymethyluracil may be an improvement [42]. 8-Hydroxyguanosine, another product of ·OH radical attack on DNA [43], might be measured [44]. Dizdaroglu [45,46] has described detailed methods for studies of the products of radical attack on DNA samples. Of course, use of such methods in humans requires access to DNA samples from the tissue suspected of being under radical attack unless one is lucky enough to find a product that is cleaved out of DNA and excreted unchanged. Leucocytes and placenta are easily available, and biopsies of liver, skin and muscle can often be obtained, but samples of other organs are difficult to obtain.

3.3. *Aromatic hydroxylation*

Under physiological conditions, attack of ·OH radical on molecules containing benzene rings results largely in formation of hydroxylated products [47]. Indeed, aromatic hydroxylation has been used as a method for measuring ·OH radical production in vitro [48,49]. Suitable non-toxic aromatic compounds for use in measuring ·OH production by whole cells and perfused organs include salicylate [50] and phenylalanine [47].

If an aromatic compound reacts with ·OH to form a specific set of hydroxylated products that can be accurately measured in body fluids or tissue extracts, and one or more of these products is not identical to enzyme-produced hydroxylated products, then formation of the 'unnatural' products could conceivably be used to assess ·OH radical formation in vivo. This assumes that the aromatic detector molecule is present at the sites of ·OH radical generation at concentrations sufficient to compete with any other molecules that might scavenge ·OH, and that any unnatural hydroxylated product is not immediately metabolised. The molecules used as 'aromatic detectors' might be 'foreign' substances administered to the subjects orally or by injection, or they might be substances present endogenously.

3.3.1. Administered substances

Under physiological conditions, attack of ·OH upon salicylate produces three products (fig.1). The major products are 2,3- and 2,5-dihydroxybenzoates, but a small amount of catechol is formed by decarboxylation [50]. 2,3-Dihydroxybenzoate has not been reported as an enzyme-produced metabolite of salicylate, although 2,5-dihydroxybenzoate has. Since low concentrations of 2,3-dihydroxybenzoate can be detected in human plasma [51] and urine [52] of normal subjects and rheumatoid patients consuming aspirin (*O*-acetylsalicylate), and there appears to be an increased formation of 2,3-dihydroxybenzoate in patients with active rheumatoid disease, in which free radical production by neutrophils is known to be

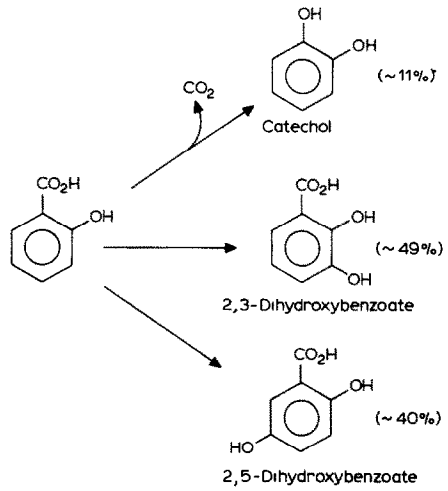

Fig.1. Products of the attack of hydroxyl radical on salicylate.

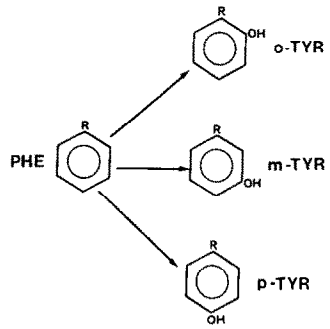

Fig.2. Products of the attack of hydroxyl radical on phenylalanine. *o*-Tyr (*ortho*-tyrosine); *m*-Tyr (*meta*-tyrosine); *p*-Tyr (*para*-tyrosine, the 'real tyrosine').

greatly increased [2], it is *possible* that 2,3-dihydroxybenzoate might be a product of ·OH attack on the salicylate molecule. It might also be produced as a minor, previously unrecognised, product of salicylate metabolism, a possibility we must always bear in mind. Many non-steroidal anti-inflammatory drugs other than aspirin contain aromatic rings, and products of attack of ·OH upon them are under investigation in our laboratory.

Attack of ·OH, generated by a Fenton system at pH 7.4, upon phenylalanine also produces three products (fig.2). Phenylalanine is less toxic to certain cell types than is salicylate, does not interfere with arachidonic acid metabolism, and is very useful for measuring ·OH production in vitro [47]. Whether it can be used in vivo is debatable; concentrations of phenylalanine normally present in human body fluids and cells are probably too low to intercept ·OH, and administration of large doses of phenylalanine to humans may be difficult to justify.

3.3.2. Endogenous substances

Determination of products of ·OH radical attack upon DNA (see above) could be classified under this heading. Another substance present in human body fluids, and thought to function as an antioxidant in vivo, is uric acid [53]. A major product of radical attack upon uric acid is allantoin [53,54]. Methods have been developed for the measurement of allantoin in human body fluids, and the results suggest an increased conversion of uric acid into allantoin during active rheumatoid disease [54]. We have recently found that different radical species give different ratios of allantoin to other oxidation products from uric acid. Determination of these other oxidation products might, we hope, give information about the specific radical species that cause uric acid oxidation in vivo.

4. CONCLUSION

Many laboratories [3–11,31,32,36,41–47,51,52,54] have recognised that further developments in our understanding of the role of free radicals in human disease require better experimental tools. A major effort is required to evaluate the many promising new techniques discussed above. The striking effects observed with SOD in protection against reoxygenation injury [57], and with metal-chelating agents [27], shows the great therapeutic potential of interfering with free radical reactions.

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